

REMARKS

Favorable reconsideration of the subject application is respectfully requested in view of the above amendments and the following remarks. Claims 1-112 are pending in the instant application, and claims 75-84 and 104 are currently under consideration. By the present amendment, claims 1-74, 85-103, and 105-112 are canceled, and claims 75, 83, 84, and 104 are amended to more specifically point out and distinctly claim certain embodiments of the invention. Support for these amendments may be found throughout the specification and claims as originally filed, and it is urged that the amendments do not constitute new matter. Support for human ANT1, ANT2, and ANT3 polypeptides having SEQ ID NOS:31, 32, and 33, respectively, is provided throughout the specification, including, *e.g.*, at page 22, lines 24-27. Support for variants having at least 95% identity to these human ANT polypeptides is provided, *e.g.*, at page 23, lines 28-30, and at page 24, lines 3-5, and support for fragments comprising at least 30 residues of these ANT polypeptides is provided, *e.g.*, at page 24, lines 6-8. It should also be noted that the above amendments are not to be construed as acquiescence with regard to the Examiner's rejections and are made without prejudice to prosecution of any subject matter removed or modified by this amendment in a related divisional, continuation or continuation-in-part application.

RESPONSE TO RESTRICTION REQUIREMENT

In response to the restriction requirement, Applicants hereby elect Group X, claims 75-84 and 104, drawn to a method of identifying an agent that binds to an ANT polypeptide and an assay plate for high throughput screening of candidate agents that bind ANT polypeptide, classified in class 435, subclass 7.1, for examination at this time.

OBJECTION TO THE DISCLOSURE

The Action objects to the disclosure for allegedly containing informalities in not providing sequence identifiers for the sequences listed in Figures 1A, 1B, and 2.

Applicants have amended the Brief Description of the Drawings to reference the appropriate sequence identifiers, as indicated above. Applicants respectfully request that this basis of objection be withdrawn in light of this amendment.

REJECTION UNDER 35 U.S.C. § 112, SECOND PARAGRAPH

Claims 75-84 and 104 stand rejected under 35 U.S.C. § 112, second paragraph, for allegedly being indefinite in their recitation of the acronym, "ANT". The Examiner indicates that this basis of rejection would be overcome by amending the independent claims to include the full name of the polypeptide followed by its acronym in parenthesis.

Applicants respectfully traverse this ground of rejection and submit that the term "ANT" is clearly defined in the claims and the specification (*see, e.g.*, page 1, lines 7-8 and page 4, line 16). Nonetheless, to expedite prosecution of the instant application, Applicants have amended independent claims 75, 83, 84, and 104 to include the full name, "adenine nucleotide translocator," as suggested by the Examiner.

Claim 84 stands rejected under 35 U.S.C. § 112, second paragraph, for allegedly being indefinite in not reciting a step of contacting the biological sample containing the ANT with the ligand in the absence of an agent. The Examiner indicates that this basis of rejection would be overcome by amending line 4 of the claim to recite that the sample is contacted with the detectable ANT ligand in the absence of a candidate agent or in the presence of the candidate agent.

Applicants respectfully traverse this rejection and submit that the skilled artisan would readily understand the claimed method. However, solely in order to expedite prosecution, and to make explicit what was implicit, Applicants have amended claim 4 essentially as suggested by the Examiner.

Applicants respectfully submit that the claims satisfy the definiteness requirements of 35 U.S.C. § 112, second paragraph and, therefore, request that this basis of rejection be withdrawn.

REJECTION UNDER 35 U.S.C. § 102(b)

Claim 104 stands rejected under 35 U.S.C. § 102(b), as allegedly anticipated by Neumann *et al.* (1994 *J. Immunol.* 152:343). More specifically, the Action asserts that Neumann *et al.* disclose 96-well ELISA plates comprising an immobilized ANT polypeptide for screening of candidate agents that bind to an ANT polypeptide, and that the immobilized ANT polypeptides of Neumann *et al.* are patentably indistinguishable from recombinant ANT polypeptides and are considered variants of recombinant ANT polypeptides of other isoforms.

Applicants respectfully traverse this rejection and submit that Neumann *et al.* fail to disclose each element of the instant claim and, therefore, fail to anticipate the claimed invention. Specifically, applicants submit that claim 104 as amended herewith is directed in pertinent part to an assay plate for high throughput screening comprising at least one immobilized recombinant human ANT polypeptide, or a recombinant variant or recombinant fragment thereof. Neumann *et al.* fail, by contrast, to describe an assay plate comprising any recombinant human ANT polypeptide. Rather, the 96-well plates described by Neumann *et al.* merely contain natural (*i.e.*, non-recombinant) ANT isolated from an abundant non-human source known to the art, namely bovine heart. Neumann *et al.* fail to disclose an assay plate for high throughput screening comprising a human ANT polypeptide, nor do Neumann *et al.* in any way contemplate human ANT in an amount suitable for use in a high throughput screen assay plate. Applicants submit that absent the disclosure of the present application, high throughput screening assay plates comprising recombinant human ANT polypeptides as recited are simply unknown to the art, and are certainly not disclosed by the cited publication, Neumann *et al.* The cited document thus cannot anticipate the subject matter of the instant claim.

Furthermore, and solely for purposes of advancing prosecution without acquiescence to this rejection, Applicants note that claim 104 has been amended to recite recombinant ANT variants having at least 95% identity to human ANT1, ANT2, or ANT3 polypeptides. Applicants submit that the bovine ANT polypeptides described by Neumann *et al.* do not have at least 95% identity to the recited recombinant human ANT1, ANT2, or ANT3 polypeptides set forth in SEQ ID NOS:31-33. It is axiomatic that to establish anticipation under 35 U.S.C. §102, each and every element of the claim must be present in a single reference.

Therefore, where Neumann *et al.* clearly fail to teach or suggest each and every element of the presently claimed invention, the cited document fails to anticipate the claimed invention.

Accordingly applicants submit that claim 104 is clearly patentable over Neumann *et al.* and respectfully request that this rejection be withdrawn.

REJECTION UNDER 35 U.S.C. § 103(a)

[A] Claims 75, 78, 79, and 83 stand rejected under 35 U.S.C. § 103(a), as allegedly obvious over Roux *et al.* (1996 *Anal. Biochem.* 234:31) in view of Adrian *et al.* (1986 *Mol. Cell. Biol.* 6:626). The Action asserts that Roux *et al.* teach a method of identifying an agent that binds to an ANT polypeptide, comprising contacting an agent “with muscle homogenate (containing cells expressing ANT or a biological sample containing ANT)”, and that Adrian *et al.* teach the recombinant expression of ANT- β -gal fusion proteins in yeast. The Action alleges that it would have been obvious to one of skill in the art at the time of the invention to improve the method of Roux *et al.* by using cells recombinantly expressing ANT polypeptide, using the protocols developed for yeast expression of Adrian *et al.* instead of muscle homogenate.

Applicants respectfully traverse these grounds for rejection and submit that the Action fails to establish a *prima facie* case of obviousness under 35 U.S.C. § 103(a). The present invention is directed in pertinent part to a method for identifying an agent that binds to an ANT polypeptide, comprising contacting a candidate agent (i) with a host cell expressing a recombinant ANT polypeptide having at least 95% identity to human ANT1, ANT2 or ANT3 as set forth in SEQ ID NOS:31-33, or (ii) with a biological sample containing at least one recombinant ANT polypeptide having at least 95% identity to human ANT1, ANT2 or ANT3 as set forth in SEQ ID NOS:31-33.

Contrary to the assertions found in the Action, Roux *et al.* and Adrian *et al.*, alone or in combination, fail to provide a person having ordinary skill in the art with the requisite reasonable expectation of successfully arriving at the claimed invention. In particular, absent the disclosure of the present application, such an ordinarily skilled artisan would have had absolutely no expectation of successfully achieving expression of any recombinant human ANT polypeptide having 95% identity to the human ANT polypeptides set forth in SEQ ID NOS:31-33. Where the cited references, alone or in combination, fail to teach or suggest each element of

the claimed invention, no *prima facie* case of obviousness is established. *In re Royka*, 490 F.2d 981 (CCPA 1974).

Roux *et al.* merely describe binding of atractyloside derivatives to mitochondria that are present in rabbit skeletal muscle homogenates, not in cells, or to mitochondria (and not cells) prepared from beef heart tissue. With regard to claims 75, 78 and 79, applicants submit that the Action errs in alleging that the muscle homogenate contains cells expressing ANT because a person having ordinary skill in the art would not understand a skeletal muscle homogenate as described by Roux *et al.* to comprise a cell. Nowhere in Roux *et al.* or in Adrian *et al.* is there any suggestion to screen for agents that bind to an ANT polypeptide by contacting a candidate agent with a cell (and for reasons addressed in greater detail below, certainly not with a cell expressing a recombinant polypeptide having 95% identity to human ANT1, ANT2 or ANT3). In particular, such a skilled artisan could not reasonably conclude, given the description of homogenate preparation by Roux *et al.* (e.g., at page 32 therein), that any cells would remain present in a sample that has been homogenized, particularly in view of steps described by Roux *et al.* such as storage in liquid nitrogen, homogenization using both a Potter homogenizer and a glass grinder, two centrifugation steps (the first removing “undisrupted fibers and heavy material”) and resuspension of pelleted material in buffered 0.3 M sucrose. Nor is this deficiency of Roux *et al.* remedied by Adrian *et al.*, who are silent with regard to any suggestion whatsoever to identify an agent that binds to an ANT polypeptide by contacting a candidate agent with a cell expressing a recombinant human ANT under conditions and for a time sufficient to permit binding of the agent to the recombinant polypeptide, as recited in pertinent part by the instant claims. In view of the combination of Roux *et al.* and Adrian *et al.*, there is thus absolutely no teaching or suggestion to practice the claimed method comprising contacting a cell (*i.e.*, a host cell expressing a recombinant human ANT polypeptide) with a candidate agent.

The Action also overstates the relevance of Roux *et al.* to *any* of the instant claims in its allegation (Action, page 13) that in view of the cited references one of ordinary skill would have been motivated “to use recombinant ANT expression as part of the method in order to allow for a greater number of candidate agents to be tested in a shorter period of time”. In fact, Roux *et al.* and Adrian *et al.* collectively fail in any way to suggest applicability of the teachings found therein to the presently claimed method, and the Action fails to point to any suggestion in these

or any other publications to screen for ANT-binding agents using recombinant human ANT polypeptides.

Roux et al. do not contemplate a screening assay for an agent that binds to an ANT polypeptide, using a recombinant human ANT polypeptide as presently recited. If anything, Roux *et al.* teach away from the instant invention. Roux *et al.* limit their disclosure to derivatives (which derivatives are selected for their detectable fluorescence properties) of atractyloside (ATR), a *known* ANT ligand, but fail to contemplate use of the assay described therein in a general method for identifying, from among candidate agents, those agents that bind ANT. Roux *et al.* also state that the “*small amount of tissue required* for a fluorometric titration assay with N-ATR or Mant-ATR allows, in addition to immunodetection, the characterization of putative ADP/ATP carrier defects *in biopsy samples from patients . . .*” (Roux *et al.*, page 37, left column, emphasis added). Applicants submit that even assuming, *arguendo*, that fluorescent ATR derivatives of Roux *et al.* were useful in screening for ANT-binding agents, the disclosure by Roux *et al.* can in no way be regarded as suggesting the subject invention method of *identifying* such agents.

In particular, Roux *et al.* allude to ANT detection using *small amounts* of tissue, or biopsy samples *from patients*, which in no way constitutes a suggestion to screen for ANT-binding agents using *any* recombinant ANT polypeptide (*e.g.*, such as that of Adrian *et al.*), because Roux et al. nowhere point to any desirability of having a recombinant ANT source, which might be needed if available samples provided insufficient amounts of ANT. Similarly, absent any evidence to the contrary, the ordinarily skilled artisan would not expect tissue or patient biopsy samples to comprise recombinant ANT, again pointing to the failure of the combined teachings of Roux *et al.* and Adrian *et al.* to suggest the present invention. Nor do Roux *et al.* anywhere suggest using a recombinant *human* ANT polypeptide, nor can any such suggestion be found elsewhere in Roux *et al.* or in Adrian *et al.* Insofar as the Action fails to point to evidence that Adrian *et al.* in any way suggest a screening method for an ANT-binding agent, much less using recombinant human ANT, the PTO impermissibly employs hindsight in view of the present application when it alleges obviousness over Roux *et al.* and Adrian *et al.*

Moreover with regard to the instant claims, applicants submit that at the time of filing the present application, a person having ordinary skill in the art would not have had a

reasonable expectation of successfully achieving recombinant expression of a polypeptide having at least 95% identity to the human ANT polypeptides set forth in SEQ ID NOS:31-33, for use in a method of identifying an agent that binds an ANT polypeptide. Additionally, the Action fails to establish that the skilled artisan, by combining the teachings of the cited references, would reasonably have had any such expectation. The Action fails to provide any specific evidence that the state of the art at the time of filing the instant application permitted recombinant expression of a human ANT polypeptide having at least 95% identity to a full length human ANT for use in a method for identifying an agent that binds to an ANT polypeptide.

Indeed, as described in the accompanying Declaration of Dr. Christen M. Andersen, recombinant production of a functional full-length recombinant human ANT polypeptide was a long-sought but elusive objective that would by no means have been regarded as a routine procedure, nor one at which the ordinarily skilled artisan would have reasonably expected to succeed. As explained by Dr. Anderson in the Declaration, at the time of filing the instant application, recombinant production of human ANT polypeptides was regarded as a particularly difficult goal that had not been successfully attained. Accordingly, Applicants submit that the skilled artisan would not have had a reasonable expectation of successfully producing the recited recombinant human ANT polypeptides, having at least 95% identity to a full length human ANT, based upon the mere expression of truncated yeast ANT fusion proteins in yeast cells described by Adrian et al. Applicants submit that where the combination of cited references fails to teach or suggest each element of the claimed invention and, further, where the combination would not provide the skilled artisan with a reasonable expectation of success, the Action fails to establish a *prima facie* case of obviousness.

Roux *et al.* are silent with regard to recombinant ANT expression, and the teachings of Adrian *et al.* are inapposite for a variety of reasons, including technical problems (see Anderson Declaration) that a person skilled in the art would expect to encounter by trying to express a recombinant human ANT polypeptide having at least 95% identity to SEQ ID NOS:31-33. By contrast, Adrian *et al.* merely describe recombinant expression in yeast of homologous (*i.e.*, yeast-derived sequences), truncated yeast ANT fusion proteins. Adrian *et al.* fail, however, to provide any suggestion whatsoever upon which an ordinarily skilled person could reasonably base an expectation of successfully expressing a recombinant human ANT polypeptide by using

a heterologous (*i.e.*, yeast) expression system. Furthermore, the disclosure of Adrian *et al.* is directed to identifying N-terminal sequences of yeast ANT polypeptides that are responsible for mitochondrial localization, in yeast cells, of yeast ANT fusion proteins which merely comprise N-terminal fragments of yeast ANT, and which in any event are not human ANT polypeptides having at least 95% identity to SEQ ID NOS:31-33. Applicants submit that Adrian *et al.* fail, however, to contemplate a method of identifying an agent that binds to an ANT polypeptide; Adrian *et al.* thus fail to suggest any desirability of combining the disclosure found therein with that of Roux *et al.* The mere fact that the teachings of the cited documents *can* be combined or modified, or that a person having ordinary skill in the art is *capable* of combining or modifying the teachings of the cited publications, does not make the resultant combination *prima facie* obvious, as the references must also suggest the desirability of the combination (*see, e.g., In re Mills*, 16 USPQ2d 1430 (Fed. Cir. 1990); *In re Fritch*, 23 USPQ2d 1780 (Fed. Cir. 1992)).

Accordingly and in view of the foregoing, applicants submit that the PTO has failed to establish a *prima facie* case of obviousness and therefore respectfully request that these rejections be withdrawn.

[B] Claims 75-79 and 83 stand rejected under 35 U.S.C. § 103(a), as allegedly being unpatentable over Roux *et al.* and Adrian *et al.*, as applied to claims 75, 78, 79, and 83 above, and further in view of Tjaden *et al.* (1998 *J. Biol. Chem.* 273:9630). The Action asserts that Tjaden *et al.* teach the recombinant expression of a plant adenine nucleotide translocator “which is considered a homologue to human ANT”, using the C43 derivative of *E. coli* strain BL21. The Action alleges that it would have been obvious to one of ordinary skill in the art at the time of the invention to modify the method of Roux *et al.* by using cells that recombinantly express an ANT polypeptide as taught by Tjaden *et al.*, and that the skilled artisan would have been motivated to do so “because it would allow for faster and easier processing of a greater number of candidate binding agents” compared to using muscle homogenate or expression in yeast cells.

Applicants respectfully traverse this ground of rejection and submit that the PTO has not established a *prima facie* case of obviousness under 35 U.S.C. § 103(a). The cited references, alone or in combination, fail to teach or suggest each element of the claimed invention, essentially for reasons set forth above in response to the rejections over Roux *et al.*

and Adrian *et al.* Additionally, applicants submit that Roux *et al.* and Adrian *et al.*, even when combined with Tjaden *et al.*, fail to teach or suggest the presently claimed method of identifying an agent that binds to an ANT polypeptide.

Applicants are puzzled by the assertion in the Action (at page 14) that the plant adenine nucleotide translocator described by Tjaden *et al.* “is considered a homologue to human ANT”, particularly where Tjaden *et al.* clearly state (at page 9630, last full paragraph) that, “[t]his carrier represents a *second type* of eucaryotic adenylate transporter *that is not related* to the adenylate translocator previously identified in mitochondria” (citing Klingenberg, 1989 *Arch. Biochem. Biophys.* 270:1-14)(emphasis added).

Applicants therefore respectfully submit that citation in the Action to use of the C43 derivative of *E. coli* strain BL21 for recombinant protein expression by Tjaden *et al.* is beside the point, where the plant ATP/ADP transporter described by Tjaden *et al.* is structurally unrelated to the human ANT polypeptides set forth in SEQ ID NOS:31-33. Therefore the skilled person would have no reason for expecting the method for recombinant expression of the plant protein described by Tjaden *et al.* to translate into successful expression of the presently recited recombinant human ANT polypeptides.

By way of contrast, Tjaden *et al.* rely on the disclosure of Miroux *et al.* (1996 *J. Mol. Biol.* 260:289) for use of the C43 derivative of *E. coli* strain BL21 as a recombinant expression host. Based on the teachings of Miroux *et al.*, however, a copy of which is enclosed for the Examiner’s convenience, applicants submit that a person having ordinary skill in the art would have understood that recombinant expression of an ANT polypeptide is hardly a routine matter. More specifically, Miroux *et al.* describe efforts to express various recombinant proteins, including mammalian ANT proteins which, unlike the plant protein of Tjaden *et al.*, are related to the human ANT proteins (SEQ ID NOS:31-33) recited by the instant claims, in a bacterial expression system. Miroux *et al.* describe multiple problems with regard to efforts to express recombinant mammalian ANT even in C43 cells, including toxicity to host cells, poor solubility of the recombinant product and accumulation of recombinant ANT in inclusion bodies (e.g., Miroux *et al.* at pages 290-291 and Table 1), which applicants submit would be recognized by those familiar with the art as a form that is not amenable to functional binding interactions with an agent that binds to an ANT polypeptide (e.g., an ANT ligand).

Applicants therefore submit that based on the teachings of Tjaden et al., who use the same expression system as Miroux *et al.*, a person having ordinary skill in the art would not expect successfully to express a recombinant polypeptide having 95% identity to a human ANT protein set forth in SEQ ID NOS:31-33. In further view of the Anderson Declaration provided herewith and discussed above, applicants therefore respectfully submit that it would be misguided to believe that the person having ordinary skill in the art at the time of the present application knew, with a reasonable expectation of success, how to arrive at the instant invention.

Because the combination of references cited by the PTO fails to teach each element of the claimed invention and, further, would not provide the skilled artisan with a reasonable expectation of success, the Action fails to establish a *prima facie* case of obviousness. Hence, and also for reasons discussed above, applicants submit that the skilled artisan would not have been motivated to combine the disclosures of Roux *et al.* pertaining to ANT ligands and Adrian *et al.* pertaining to yeast ANT fusion proteins, with bacterial cells that recombinantly express a plant polypeptide unrelated to human ANT (SEQ ID NOS:31-33) as taught by Tjaden *et al.*, to arrive at the present invention.

Moreover, and as also noted above, the mere fact that the teachings of the cited documents *can* be combined or modified, or that a person having ordinary skill in the art is *capable* of combining or modifying the teachings of the cited documents, does not make the resultant combination *prima facie* obvious, as the references must also suggest the desirability of the combination (*see, e.g., In re Mills*, 16 USPQ2d 1430 (Fed. Cir. 1990); *In re Fritch*, 23 USPQ2d 1780 (Fed. Cir. 1992)). Thus, even assuming, *arguendo*, that the ordinarily skilled artisan might have been motivated to try expressing recombinant ANT polypeptides other than the polypeptides described in the references cited by the PTO, nowhere do the cited publications teach or suggest expressing the specifically recited polypeptides having at least 95% identity to the human ANT polypeptides set forth in SEQ ID NOS:31-33. Applicants therefore submit that no *prima facie* case of obviousness has been established.

Accordingly, applicants respectfully request that the rejections under 35 U.S.C. § 103(a) be withdrawn in light of the above amendments and remarks.

Additionally, applicants wish to call the Examiner's attention to several related co-pending applications having claims potentially directed to similar subject matter. Reference to the appended "Table of Co-Pending Applications" is therefore requested.

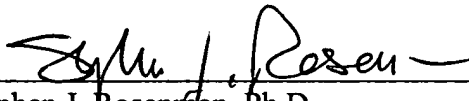
The Commissioner is authorized to charge any additional fees due by way of this Amendment, or credit any overpayment, to our Deposit Account No. 19-1090.

Applicants submit that all of the claims remaining in the application are allowable. Favorable consideration and a Notice of Allowance are earnestly solicited.

Respectfully submitted,

Christen M. Anderson et al.

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SJR:kw

Enclosures:

Postcard
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APPENDIX: TABLE OF CO-PENDING APPLICATIONS

U.S.A.N.	Atty. Docket No.	Examiner	Claims directed to ____ (Comments)
09/393,441 (present application)	660088.420C1	Sheridan Snedden	__ isolated recombinant huANT3 polypeptide that localizes to mitochondrial membrane Statutory double-patenting rejection of claims 42, 46-48, 51 and 57 over claims 42, 46-48, 51 and 57 of 09/185,904
09/185,904	660088.420	Holly G. Schnizer	__ isolated recombinant huANT3 polypeptide Obviousness-type double patenting rejection of claims 42, 46-50 over claims 42, 46-48, 51 and 57 of 09/393,441
09/811,131	660088.420D1	Holly G. Schnizer	__ method of identifying agent that binds to ANT polypeptide
09/811,185	660088.420D2	Rebecca L. Anderson	__ method of treatment using ANT ligand
09/810,644	660088.420D3	Rebecca L. Anderson	__ ANT ligand
09/811,094	660088.420D4	Holly G. Schnizer	__ recombinant expression construct, host cell, and method of making recombinant ANT polypeptides and fusion proteins
09/811,132	660088.420D5	Holly G. Schnizer	__ methods of detecting and isolating an ANT polypeptide, using ANT ligand
09/809,827	660088.420D6	Holly G. Schnizer	__ isolated recombinant huANT1 polypeptide
09/809,889	660088.420D7	Holly G. Schnizer	__ isolated recombinant huANT2 polypeptide
09/569,327	660088.443	Sheridan Snedden	__ method of producing recombinant ANT polypeptides and fusion proteins using tightly regulated promoter
10/684,232	660088.433C2	(none assigned)	__ ANT-energy transfer peptide fusion proteins



COMMUNICATION

Over-production of Proteins in *Escherichia coli*: Mutant Hosts that Allow Synthesis of some Membrane Proteins and Globular Proteins at High Levels

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We have investigated the over-production of seven membrane proteins in an *Escherichia coli*-bacteriophage T7 RNA polymerase expression system. In all seven cases, when expression of the target membrane protein was induced, most of the BL21(DE3) host cells died. Similar effects were also observed with expression vectors for ten globular proteins. Therefore, protein over-production in this expression system is either limited or prevented by bacterial cell death. From the few survivors of BL21(DE3) expressing the oxoglutarate-malate carrier protein from mitochondrial membranes, a mutant host C41(DE3) was selected that grew to high saturation cell density, and produced the protein as inclusion bodies at an elevated level without toxic effect. Some proteins that were expressed poorly in BL21(DE3), and others where the toxicity of the expression plasmids prevented transformation into this host, were also over-produced successfully in C41(DE3). The examples include globular proteins as well as membrane proteins, and therefore, strain C41(DE3) is generally superior to BL21(DE3) as a host for protein over-expression. However, the toxicity of over-expression of some of the membrane proteins persisted partially in strain C41(DE3). Therefore, a double mutant host C43(DE3) was selected from C41(DE3) cells containing the expression plasmid for subunit b of bacterial F-ATPase. In strain C43(DE3), both subunits b and c of the F-ATPase, an alanine-H⁺ symporter, and the ADP/ATP and the phosphate carriers from mitochondria were all over-produced. The transcription of the gene for the OGCP and subunit b was lower in C41(DE3) and C43(DE3), respectively, than in BL21(DE3). In C43(DE3), the onset of transcription of the gene for subunit b was delayed after induction, and the over-produced protein was incorporated into the membrane. The procedure used for selection of C41(DE3) and C43(DE3) could be employed to tailor expression hosts in order to overcome other toxic effects associated with over-expression.

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Keywords: *E. coli*; T7 RNA polymerase; over-expression; membrane proteins

Abbreviations used: OGCP, oxoglutarate-malate transport protein from mitochondria; F-ATPase, H⁺-transporting F₁F₀-ATPase; OSCP, oligomycin sensitivity conferral protein, a subunit of bovine F-ATPase; GFP, green fluorescent protein from the jelly-fish, *Aequoria victoria*; IPTG, isopropyl-2- α -thio-galactopyranoside; PCR, polymerase chain reaction; SDS-PAGE, sodium dodecyl sulphate-polyacrylamide gel electrophoresis; expression plasmid names consist of the name of the plasmid vector, followed (in parentheses) by the recombinant they encode.

Escherichia coli is one of the most successful vehicles for over-expression† of both prokaryotic and eukaryotic proteins (for reviews see Hockney, 1994; Grishammer & Tate, 1995). However, the

† Here, the term "over-expression" implies that the target protein is expressed in the *E. coli* cells at a level that would provide a convenient source of material for structural studies. Minimally this is likely to be about 1 mg of the target protein per litre of bacterial culture.

expression vectors for many membrane proteins, as well as for some cytoplasmic proteins (Dong *et al.*, 1995), for cell division proteins (de Boer *et al.*, 1988; Gutzman *et al.*, 1992) and for other toxic proteins such as DNase (Doherty *et al.*, 1993) kill the host bacterium. Therefore, we have investigated the toxicity of over-expression of membrane proteins in *E. coli* in one of the most widely used expression systems, in which the target gene is transcribed from the vector by bacteriophage T7 RNA polymerase (Studier *et al.*, 1990). In the *E. coli* BL21(DE3) host strain, used in conjunction with pET vectors, the T7 RNA polymerase is produced from the λ -lysogen DE3 in the host bacterium, and its expression is under the control of the IPTG-inducible lac UV5 promoter. This system has been employed successfully for over-production of many globular proteins, but in many other cases significant over-production cannot be achieved because of the toxicity of over-expression (Studier *et al.*, 1990; George *et al.*, 1994).

The toxic effects of the over-expression of seven membrane proteins (see Table 1) cloned in pET and

related expression plasmids towards the *E. coli* BL21(DE3) host cells were investigated by attempting to grow cells containing the plasmids on two sets of agar plates, one containing IPTG and the other lacking the inducer. The proteins investigated were the OGCP, the phosphate carrier and the ADP/ATP carrier, all three being members of a super-family of transport proteins with six transmembrane spans (Walker & Runswick, 1993), subunits b and c of the *E. coli* F-ATPase with one and two transmembrane spans, respectively (Fillingame, 1990), and a fusion protein between bacteriophage T7 10a protein and the alanine-H⁺ carrier from *Bacillus* PS3, which is thought to have 10 to 12 transmembrane α -helices (Kamata *et al.*, 1992). In earlier studies, the OGCP had been shown to be over-produced at high levels (10 mg per litre of bacterial culture) in *E. coli* BL21(DE3) (Fiermont *et al.*, 1993). None of the seven vectors produced colonies on the plates containing IPTG, and in the absence of IPTG only very small colonies formed from cells containing plasmids for the b-subunit of *E. coli* F-ATPase, for the bovine ADP/ATP carrier,

Table 1. Expression of various proteins in BL21(DE3), C41(DE3) and C43(DE3) hosts

Protein*	Location ^b	Expression level ^c		
		BL21	C41	C43
Bovine OGCP (m)				
Bovine phosphate carrier (m)	IB	10 ¹	100 ¹	84 ¹
Bovine ADP/ATP translocase (m)	IB	5 ¹	35 ¹	52 ¹
<i>Bacillus</i> PS3 alanine/H ⁺ carrier (m)	IB	—	9 ¹	18 ¹
<i>E. coli</i> F-ATPase subunit b (m)	IB	—	19 ²	79 ²
<i>E. coli</i> F-ATPase subunit c (m)	IB/M	—	8 ²	25 ²
Bovine F-ATPase subunit b (m)	M	2 ²	10 ²	15 ²
Bovine F-ATPase subunit α (g)	IB	ND	30 ^{2,3}	ND
Bovine F-ATPase subunit β (g)	IB	35 ²	135 ²	ND
Bovine F-ATPase subunit γ (g)	IB	50 ²	240 ²	ND
Bovine F-ATPase subunit δ (g)	IB	11 ²	74 ²	ND
Bovine F-ATPase subunit d (g)	IB	4 ²	18 ²	ND
Bovine F-ATPase subunit OGCP (g)	IB	10 ²	20 ²	3 ²
Bovine F-ATPase subunit F ₀ (g)	IB	50 ¹	300 ¹	ND
Bovine F-ATPase inhibitor protein (g)	C	65 ²	130 ²	ND
<i>D. melanogaster</i> staufen protein (g)	C	8 ²	70 ²	ND
<i>Aequoria victoria</i> GFP (g)	C	—	ND ⁴	ND
	IB/C	37 ²	140 ²	ND

At the end of the expression experiment (three hours growth in BL21, 18 hours in C41 in most cases), cells were centrifuged (7000 g, 10 minutes) and re-suspended in buffer (20 ml) consisting of 10 mM Tris-HCl (pH 8.0), 1 mM EDTA and 0.001% (w/v) phenylmethylsulphonyl fluoride. Bacteria containing over-produced proteins were passed twice through a French pressure cell (pre-cooled to 4°C), inclusion bodies were collected by centrifugation (10 minutes, 10,000 g), and the membrane and cytosolic fractions were separated by ultra-centrifugation (two hours, 100,000 g).

* m, membrane protein, g, globular protein.

^b IB, inclusion bodies; C, soluble in cytosol; M, in membrane; for *E. coli* F-ATPase subunit b, IB/M indicates that, C41(DE3), the protein was in a form that was difficult to solubilize in detergent, but in C43(DE3) it was in the membrane and was readily detergent extractable (see the text); for the GFP, IB/C indicates that the protein was partially soluble and partially in inclusion bodies in both BL21(DE3) and C41(DE3).

^c The expression level is given as mg protein/litre of bacterial cells, quantified by: ¹ bicinchoninic acid assay, or ² N-terminal sequencing. A portion of cells was solubilized in 1% SDS, and the proteins were separated by SDS-PAGE (Laemmli, 1970), transferred to polyvinylidene difluoride membranes and stained with PAGE 83 dye. Appropriate bands were excised and introduced into the sequencer. The yields of phenylthiohydantoins released at each of 15 consecutive cycles of Edman degradation were measured by HPLC, and the amount of the protein of interest on the membrane was estimated by extrapolation to cycle zero. Each experiment was performed twice. ³ The bovine F-ATPase b-subunit probably has two trans-membrane spanning α -helices and is not related in sequence to the *E. coli* b-subunit, which has one trans-membrane span (Walker *et al.*, 1987); the staufen protein (St Johnston *et al.*, 1991) was detected in the soluble fraction of the cells by Western blotting by D. St Johnston. A hyphen indicates that because of toxicity of the expression plasmid, no expression was obtained. ND, not determined. With the exceptions of the alanine-H⁺ carrier, which was cloned in pCGT180 (kindly donated by Dr C. G. Tate, it was derived from pGEMX and produces a fusion protein with the major capsid protein 10A of phage T7), and the staufen protein, which was cloned into pET7, the coding sequences of the various proteins were cloned into pMW7 (Way *et al.*, 1990). Also, see Collinson *et al.* (1994) and Orriss *et al.* (1996) for more details of vectors, and Chalfe *et al.* (1994) for details of the GFP. The GFP was cloned from a mutant, GFPA (K. Siemerling, R. Goblik & J. Haseloff, unpublished results).

and for the alanine-H⁺ carrier. The cells in these very small colonies were not viable, and therefore it would not be possible to produce inocula for over-expression cultures in liquid media. Small viable colonies were obtained with the plasmid for *E. coli* subunit c. Similar experiments were conducted with vectors for ten globular proteins (see Table 1); none of them formed colonies in the presence of IPTG. Therefore, all 17 of the expression plasmids that were examined produced toxic effects on the BL21(DE3) host, with a wide spectrum of severity. The plasmids encoding membrane proteins were the most toxic, but among plasmids encoding membrane proteins, the one encoding the OGCP was the least toxic.

A control experiment was conducted with five different expression vectors from the pET family, all of them lacking a target gene for possible over-expression. They were pMW7, pET17b (containing an N-terminal T7 tag), pET23a (containing a N-terminal T7 tag and a C-terminal His-tag), pET29a (containing an S-tag) and pGEMEX-1 (containing a gene 10a fragment). Surprisingly, none of the cells containing the "empty" plasmids produced colonies in the presence of IPTG, except for pET 17b, which gave very small colonies, demonstrating that the plasmids themselves are intrinsically toxic to *E. coli* BL21(DE3) host cells. Therefore, the toxicities for expression plasmids containing target genes, consist of contributions from both the vector (and associated tags) and the target gene.

The inhibitory effects of the pMW7 (OGCP) expression vector on *E. coli* BL21(DE3) were also studied in liquid culture containing ampicillin. The culture was grown for four hours before addition of the inducer, IPTG (see phase 1 in Figure 1A). One hour or so later, the cells had stopped dividing and the absorbance of the culture decreased (phase 2). After a further five to six hours, the absorbance rose again (phase 3) and eventually reached a value greater than 5. The maximal level of expression of the OGCP was attained three hours after addition of inducer, and it diminished thereafter. Therefore, phase 3 corresponds to the outgrowth of cells that had lost the ability to express the target gene. Similar three-phase growth curves have been observed in our studies of all of the proteins that have been over-produced in *E. coli* BL21(DE3). Some variation was observed in the length of the lag in phase 1 from three hours (GFP) to ten hours (*E. coli* F-ATPase subunit c), and in maximal cell density in phase 2 from 0.5 to 2.0, depending on the degree of toxicity associated with the plasmid.

The toxicity mediated by IPTG induction of OGCP expression in *E. coli* BL21(DE3) in a liquid culture was investigated by plating the cells in the absence of selection pressure, in the presence of ampicillin, and in presence of both ampicillin and IPTG. After 30 minutes, the number of viable cells had decreased dramatically from 10^8 to 10^4 even in the absence of ampicillin (see Figure 1B). The absorbance of the culture was still increasing at this

juncture, and so the cells were dying but had not lysed. In the residual viable population, only 10% of the bacteria retained ampicillin resistance two hours after induction, and three hours later only 1% of the population was resistant to the antibiotic. Eight hours after induction, the number of viable cells equalled the number of cells calculated from the absorbance, showing that the culture now contained predominantly cells that had lost the plasmid.

The ampicillin-resistant cells were also resistant to IPTG (Figure 1B), and the colonies contained two sub-populations of larger and smaller sizes (Figure 1C). Neither of these phenomena has been described in previous investigations of this expression system (Studier *et al.*, 1990). In a separate control experiment, where no IPTG was added to the liquid culture, the plasmid was stable, and the number of viable cells was similar to the number of cells calculated from the absorbance. However, on plates, a small fraction of the population was again resistant to IPTG. In the uninduced liquid culture, the ratio of cells resistant to IPTG compared to the total number of cells was stable at around 3×10^{-5} , whereas induction of expression by addition of IPTG increased this ratio to 3×10^{-3} (Figure 1D). Therefore, the expression of the OGCP in *E. coli* BL21(DE3) grown in liquid cultures had apparently increased the number of colonies resistant to IPTG. This effect of IPTG is difficult to quantify as its addition to the culture leads to the death of wild-type cells, thereby selecting for mutants, but it remains possible that the induction of expression of the protein provokes an SOS response in the host (Studier *et al.*, 1990; Murli & Walker, 1993; Friedberg *et al.*, 1995).

This apparent increase in the frequency of mutants by over-expression of the OGCP on *E. coli* BL21(DE3) presented the opportunity to select mutant host strains that might be more tolerant to over-expression of the OGCP. The first round of selection was conducted with *E. coli* BL21(DE3) transformed with pMW7(OGCP). Four hours after induction, a 100-fold dilution of cells from the culture was plated on solid medium containing IPTG, giving sub-populations of large and small colonies as before (Figure 1C). Three large colonies and one small colony were examined for their ability to express the OGCP in liquid media. No OGCP was produced by cells grown from the large colonies, but a culture grown from the small colony was found to produce the OGCP and to continue growing in the presence of IPTG, eventually attaining a saturation absorbance similar to control cultures grown in the absence of inducer. The strain of cells from the small colony was named *E. coli* C41(DE3). Its phenotype was stable; it continued to give rise to small colonies in the presence of IPTG (Figure 2A), and to grow and to produce the OGCP in liquid cultures in the presence of inducer.

Plasmid pMW7(OGCP) was re-isolated from cells of *E. coli* C41(DE3), and was transformed back into *E. coli* BL21(DE3), restoring the toxic phenotype.

292

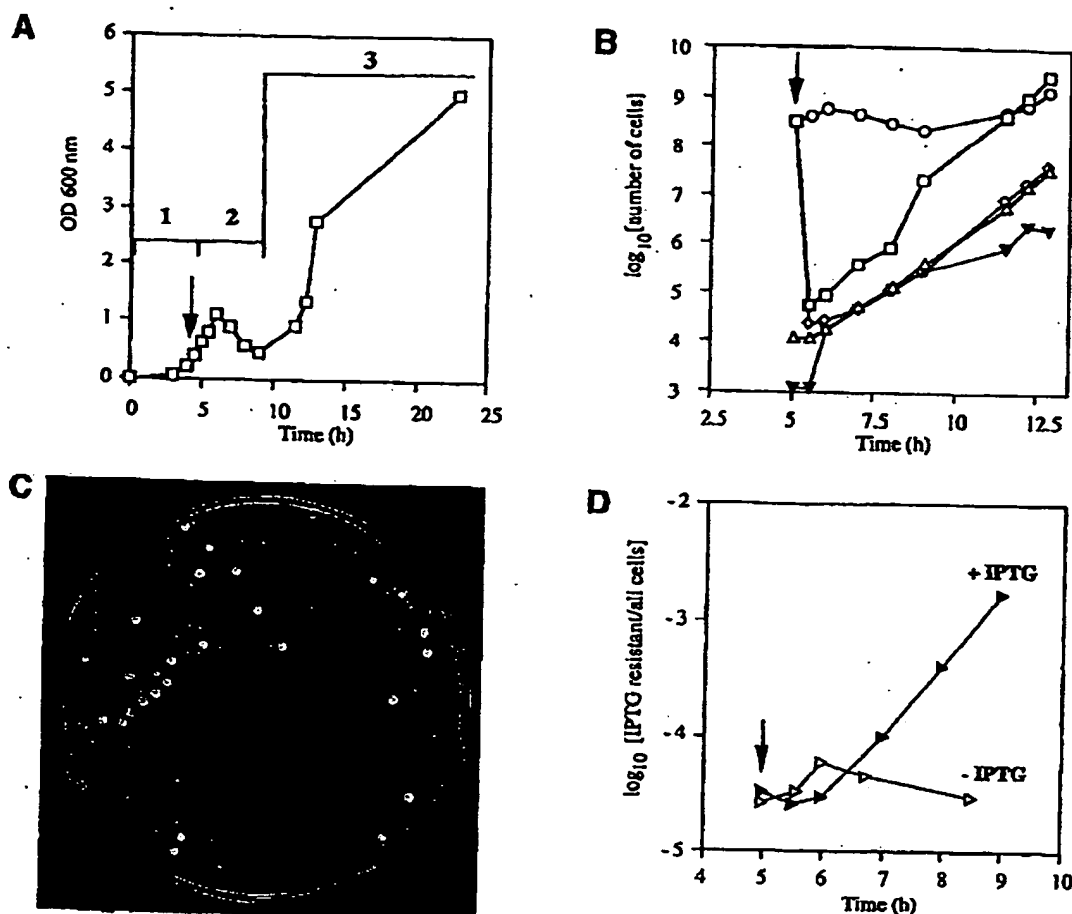
Protein Over-expression in *E. coli*


Figure 1. Effect of expression of bovine OGCP on the growth of *E. coli* BL21(DE3) host cells. In A, B and D, the vertical arrow indicates the addition of the inducer IPTG (final concentration 0.7 mM) to the liquid culture. A, A fresh colony of the host containing the plasmid pMW7(OGCP) was inoculated into 2 × TY medium (50 ml) supplemented with ampicillin (final concentration 50 µg/ml). Three phases of growth are marked: 1, pre-induction; 2, post-induction cell death; 3, overgrowth of the culture. B to D, Analysis of the bacterial population after induction of expression of the bovine OGCP. Portions (100 µl) of each dilution (1 in 10, 1 in 10², 1 in 10³, 1 in 10⁴ and 1 in 10⁵) were spread on three sets of agar plates, (0.7 mM, final concentration), IPTG and ampicillin (50 µg/ml), ampicillin alone, and no additives, respectively. The number of viable cells was determined by counting the colonies on the most suitable plate with 100 to 300 colonies per plate. In B, the analysis was performed on samples from A. The symbols used are as follows: (○) Number of cells calculated from the absorbance; (□) number of viable cells on 2 × TY plates; (◇) ampicillin-resistant colonies; (△) ampicillin and IPTG-resistant colonies; (▽) small colonies resistant to both ampicillin and IPTG ("small" colonies). C, Large and small colony formation in the presence of ampicillin and IPTG of a sample of cells from a liquid culture taken 11.5 hours after induction by IPTG. D, The frequency of ampicillin and IPTG resistant colonies compared with the total population: (▷) non-induced culture; (◀) induced culture.

Strain *E. coli* C41(DE3) was cured of pMW7(OGCP) by growth in liquid medium in the absence of ampicillin. Each day, a portion of the culture was diluted 1000-fold, and plated out in the presence of IPTG and in the absence of ampicillin. After seven days, a large colony lacking the plasmid arose. Retransformation of cells from this colony with pMW7(OGCP) restored the ability to grow in the presence of IPTG in liquid culture and to over-express the OGCP. Therefore, the

mutation affecting over-expression of the OGCP is in strain C41(DE3), and not in the plasmid pMW7(OGCP).

Subsequently, it has proved to be possible to over-express many other proteins without toxic effects in *E. coli* C41(DE3) (see below). However, the toxicity of over-expression of other proteins, including the b subunit of *E. coli* F-ATPase, persisted in strain C41(DE3). Therefore, a second round of selection was conducted on *E. coli*

Protein Over-expression in *E. coli*

293

C41(DE3) transformed with pMW7(Ecb). From 15 small colonies arising by plating in the presence of IPTG, one was found that over-expressed subunit b and continued to grow after induction. This strain was named *E. coli* C43(DE3), and the additional mutation was again shown to be associated with the bacterial genome. Similar to strain C41(DE3), the phenotype of C43(DE3) is stable (see Figure 3A). In contrast to their toxic effects on *E. coli* BL21(DE3),

"empty" plasmids do not inhibit the growth of either the C41(DE3) or C43(DE3) strains.

It should be emphasized that the number of small colonies and the proportion of those small colonies that are competent for expression of a target protein, differ widely according to the toxicity of the expression plasmid. With relatively non-toxic plasmids such as pMW7(OGCP) and pMW7(GFP), encoding the green fluorescent protein, small

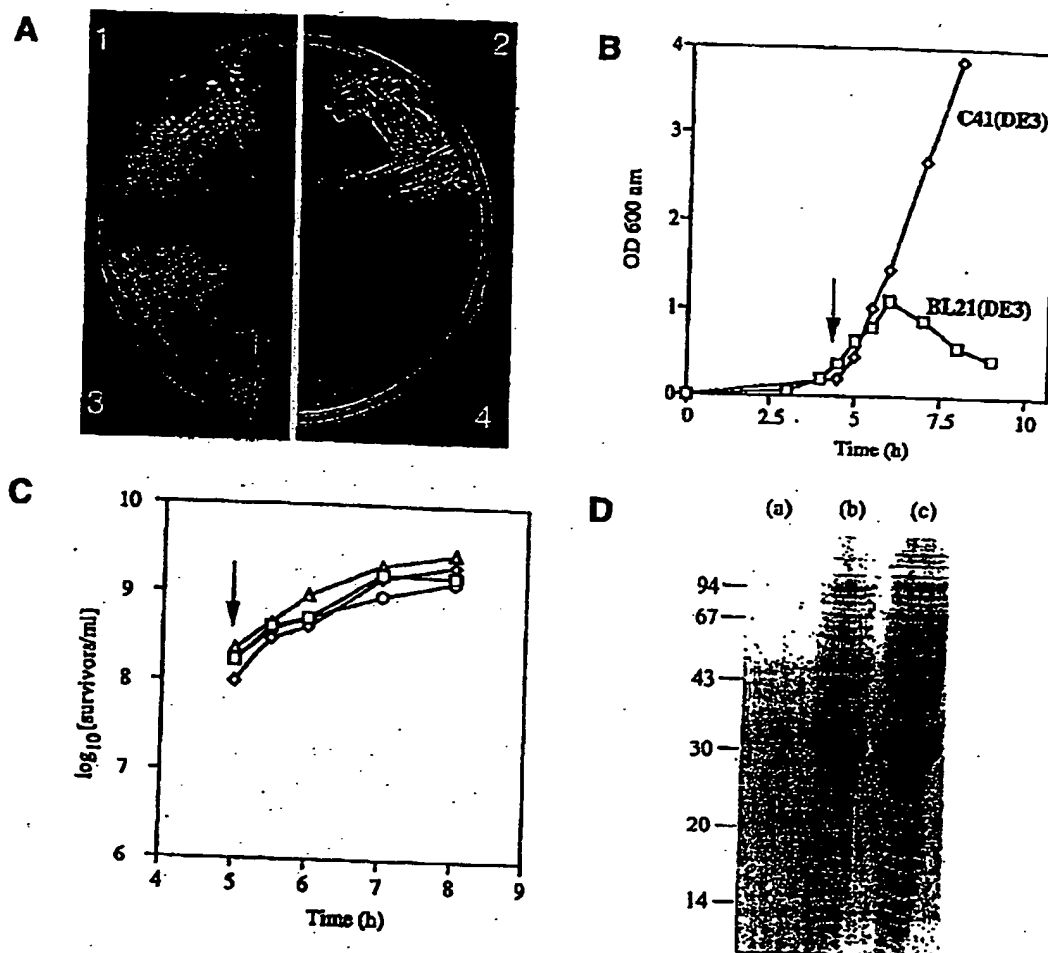


Figure 2. Comparison of the expression of the OGCP in *E. coli* BL21(DE3) and C41(DE3) hosts. A, Comparison of phenotypes of *E. coli* BL21(DE3) and mutant C41(DE3), both containing pMW7(OGCP). Quadrants 1 and 2, *E. coli* C41, in the absence and presence of IPTG, respectively; quadrants 3 and 4, *E. coli* BL21(DE3) in the absence and presence of IPTG, respectively. B, Growth of the two strains containing pMW7(OGCP) in the liquid culture (for details, see the legend to Figure 2). The arrows in B and C indicate the addition of inducer. (O) Number of cells calculated from the absorbance; (□) number of cells able to grow on 2 × TY plates; (◇) ampicillin resistant colonies; (Δ) colonies resistant to both ampicillin and IPTG. C, Analysis of the bacterial population by SDS-PAGE. The cultures were grown in 250 ml of broth. In both cases, the protein formed inclusion bodies in bacterial cytoplasm. They were each re-suspended in 4 ml of buffer and 1 μl was analysed on the gel, which was stained with PAGE 83 dye. At the left-hand side, the positions of molecular mass markers are indicated (in kDa). Lane (a), OGCP expressed in *E. coli* BL21(DE3) three hours after induction; lane (b), OGCP expressed in C41(DE3) three hours after induction in medium lacking ampicillin; lane (c), OGCP expressed in C41(DE3) 18 hours after induction by IPTG added at the starting point of the culture.

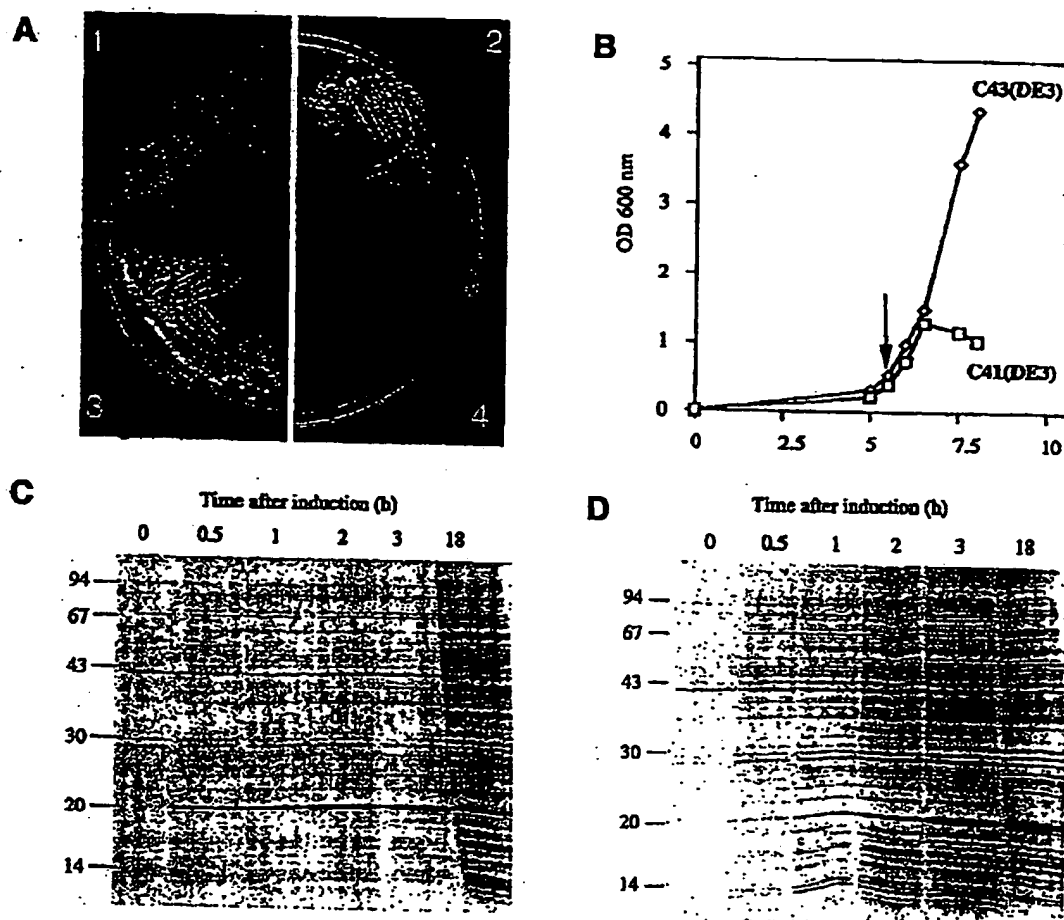


Figure 3. Comparison of the expression of subunit b of *E. coli* F-ATPase in *E. coli* C41(DE3) and C43(DE3) hosts. Freshly transformed colonies C41(DE3) and C43(DE3) each containing pMW7(Ecb) were inoculated into 2 x TY medium (50 ml) and grown at 37°C. A, Comparison of phenotypes of *E. coli* C41(DE3) and mutant C43(DE3), both containing pMW7(Ecb). Quadrants 1 and 2, *E. coli* C43(DE3), in the absence and presence of IPTG, respectively; quadrants 3 and 4, *E. coli* C41(DE3) in the absence and presence of IPTG, respectively. B, Growth curves of *E. coli* C41(DE3) and C43(DE3) expressing subunit b. The arrow indicates the induction of expression by IPTG. C and D, SDS-PAGE analysis of the expression of the b-subunit in *E. coli* C41(DE3) and C43(DE3), respectively. The equivalent of 5 µl of culture was analysed at the times indicated above each slot. On the left-hand side, the migration positions of standard proteins are indicated. The gel was stained with Coomassie 83 dye.

colonies competent for over-expression were common and easily identifiable, whereas with more toxic plasmids such as pMW7(Ecb), mutants of C41(DE3) expressing subunit b were rare.

The advantages of strains C41(DE3) and C43(DE3) as hosts for over-expression of the OGCP and subunit b of the F-ATPase, respectively, are illustrated in Figures 2 and 3. Both parental strains stopped growing after induction of expression, whereas the mutant hosts continued to grow to high cell densities (see Figures 2B and 3B). By analysis of the cell population in the culture after induction of over-expression (Figure 2C), it was apparent that pMW7(OGCP) remained stable in strain C41(DE3), and in addition, the number of

viable cells correlated with the number of cells calculated from the absorbance. In C41(DE3), at least ten times more OGCP was expressed than in BL21(DE3) (Figure 2D, lanes (a) and (b)). Moreover, strain C41(DE3) containing pMW7(OGCP) could be grown in 2 x TY broth, containing IPTG but lacking ampicillin, without overgrowth (Figure 2D, lane (c)). The final cell density in C41(DE3) was six times greater than in BL21(DE3), and therefore the amount of OGCP produced per cell is somewhat higher in C41(DE3) than in BL21(DE3).

The course of expression of the *E. coli* F-ATPase b subunit in C41(DE3) and C43(DE3) differed (see Figure 3C and D), the onset of protein production being delayed in C43(DE3) by about one hour

relative to C41(DE3). Three hours after induction, three times more protein had been produced in C43(DE3) than in C41(DE3); 15 hours later the amount of subunit b in C41(DE3) had decreased because the culture had become overgrown by cells that had lost expression capacity, as discussed above. Less subunit b was produced per cell in C43(DE3) than in C41(DE3), but the global amount of protein produced per litre of culture was higher in C43(DE3) because the cells continued to divide after induction of expression.

Transcription of the gene for the OGCP was compared in BL21(DE3) and C41(DE3) hosts (see Figure 4). The main RNA band had migrated further than 16 S ribosomal RNA to a position corresponding to an mRNA of about one kilobase, as expected for the OGCP. Three larger RNAs, also

detected with the OGCP probe, probably arise by the T7 RNA polymerase transcribing beyond the T7 transcriptional terminator, which is immediately after the OGCP gene in the plasmid. In longer exposures of the blot (not shown), similar bands could be seen in C41(DE3) also, but in relatively lower amounts compared with the main band in BL21(DE3). The basal level of OGCP mRNA synthesis in BL21(DE3) was five times higher than in C41(DE3) (see Figure 4B), and the maximal amount of OGCP mRNA synthesized after induction was about ten times greater in BL21(DE3) than in C41(DE3). Moreover, the maximum amount of OGCP mRNA appears to have been reached after 45 minutes in BL21(DE3) and at least 45 minutes later in C41(DE3).

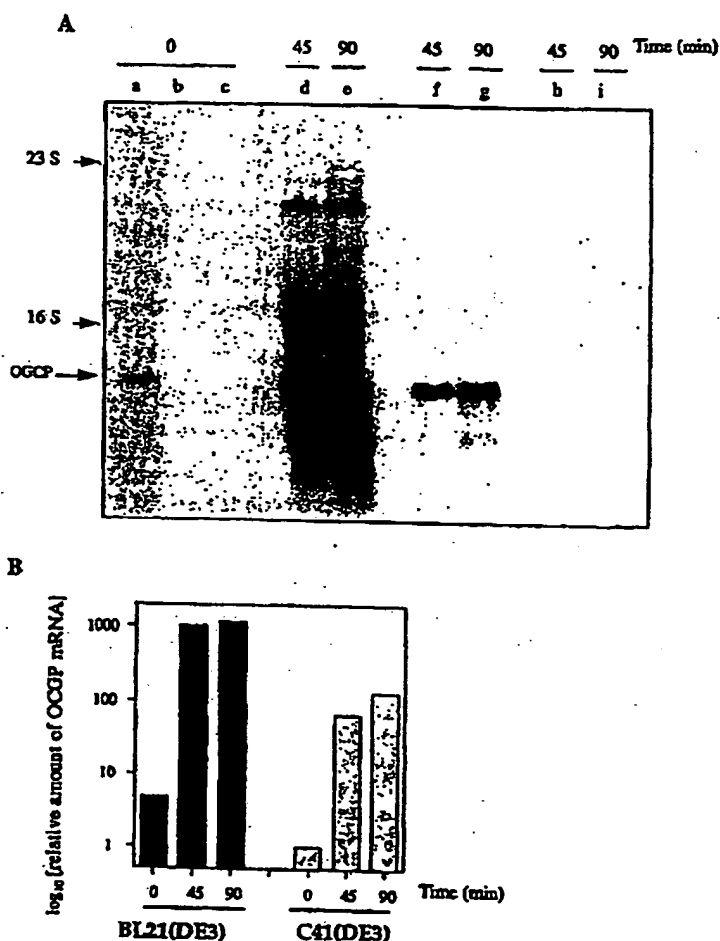


Figure 4. Analysis of transcripts of the OGCP in *E. coli* BL21(DE3) and C41(DE3). RNA samples from cells of *E. coli* BL21(DE3) and C41(DE3) (4 ml), both containing the expression plasmids for the OGCP and subunit b of *E. coli* F-ATPase, were prepared according to Ausubel *et al.* (1987) and Uzan *et al.* (1988), respectively. RNA (3 µg) was fractionated by electrophoresis under denaturing conditions in a 1% agarose gel, and then transferred to a Hybond-N membrane. Pre-hybridization and hybridization of the membrane were carried out for 18 hours at 42°C. The DNA probe for the bovine OGCP, corresponding to its complete coding sequence, was amplified from a plasmid by PCR, and radio-labelled with [α -³²P]dCTP (50 µCi) by use of an oligonucleotide labelling kit (Pharmacia Biotech Ltd, St Albans, Herts, AL1 3AW, UK). The membrane was hybridized in the presence of the probe, washed twice at 42°C in 2×SSC buffer containing 0.1% SDS, and twice at 65°C in 0.1×SSC buffer containing 0.1% SDS (SSC buffer consists of 3 M sodium chloride and 0.3 M sodium citrate (pH 6.5)). The radioactivity on the membrane was measured by densitometry with a computing densitometer (Molecular Dynamics, model 300A with ImageQuant version 3.2 software) of a radioautograph exposed to Fuji RX film. A, Autoradiograph of the membrane after 15 minutes; exposure. Lanes a, d and e, RNA samples of

BL21(DE3) expressing the OGCP; lanes b, f, and g, RNA samples of C41 expressing the OGCP; lanes c, h and i, control RNA samples from C41 expressing the *E. coli* F-ATPase b-subunit. Samples were taken at various times after induction, as shown on top. The migration positions of the OGCP mRNA, and of the 16 S and 23 S ribosomal RNAs are indicated on the left. B, Relative amounts of the OGCP mRNA, estimated by densitometry of the appropriate bands on two different exposures of the membrane. In C41(DE3), the signal at time zero was chosen as reference.

296

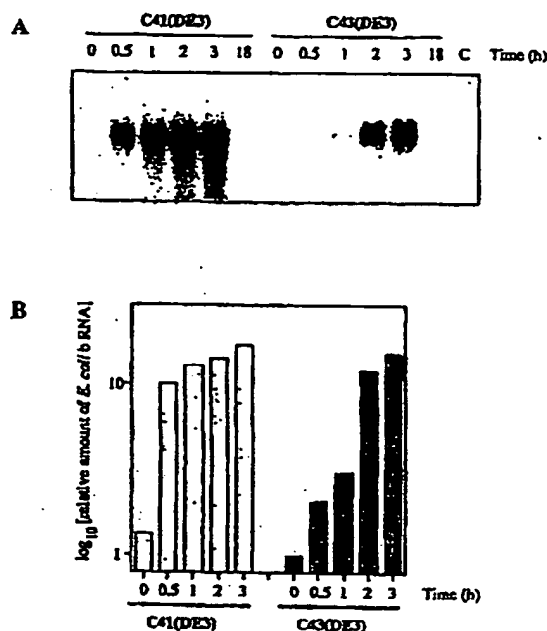
Protein Over-expression in *E. coli*

Figure 5. Analysis of transcripts of *E. coli* F-ATPase b subunit in C41(DE3) and C43(DE3). For experimental details, see legend to Figure 4. After hybridization of RNA samples with the probe consisting of the entire coding sequence of the b subunit, the membrane was exposed to an image plate for 18 hours, and the radioactivity was measured with a PhosphorImager (Molecular Dynamics, Chesham, Bucks, HP5 2PX, UK). A, Autoradiography of RNA samples from C41(DE3) and C43(DE3) at various times (shown on top) after induction of expression of F-ATPase subunit b. Lane C contains a control sample of RNA from C41(DE3) cells in which the OGCP had been over-expressed. B, Quantification of the mRNA samples in A. In C43(DE3), the signal at time zero was chosen as reference.

A similar comparison of the expression of the b subunit of the *E. coli* F-ATPase in C41(DE3) and C43(DE3) was also conducted (Figure 5). The final amount of mRNA for *E. coli* F-ATPase subunit b accumulated per cell after three hours was approximately the same in both strains, but the maximal level of this mRNA was attained in 30 minutes after induction in C41(DE3) and in two hours in C43(DE3). The basal level of expression was slightly lower in C43(DE3) than in C41(DE3).

These studies indicate strongly that a major component of toxicity of protein over-expression in *E. coli*-T7 RNA polymerase systems is linked to transcription of the target gene and, together with published work discussed below, they suggest that the toxicity probably arises from the uncoupling of transcription from translation. The T7 RNA polymerase transcribes DNA about seven times faster than *E. coli* RNA polymerase, and therefore

transcription outstrips translation, permitting unstable naked RNA stretches to form (Iost & Dreyfus, 1995; Makarova *et al.*, 1995). By an unknown mechanism, over-expression of either β -galactosidase or an inactive form of elongation factor Tu have been shown to lead to destruction of ribosomal RNAs, and the ensuing lethal effects of over-expression (Dong *et al.*, 1995). Experiments described above are consistent with this mechanism of lethality of protein over-expression. Strain BL21(DE3) containing the expression plasmid pMW7(OGCP) produced a large amount of the cognate mRNA from the plasmid, whilst at the same time the target protein was present in the cells at rather low levels. In contrast, in strain C41(DE3) the same transcript was made more slowly, and despite the maximal level being ten times lower than in BL21(DE3), more of the target protein was synthesized in C41(DE3). Similar effects were noted by comparison of the expression of subunit b of *E. coli* F-ATPase in C41(DE3) and C43(DE3).

At present, the locations of the mutations in C41(DE3) and C43(DE3) are not known, but plausible hypotheses concerning the C41(DE3) mutation are that either it affects the activity of the T7 RNA polymerase or that it reduces the amount of polymerase produced. Both effects would probably help to prevent uncoupling of transcription and translation. It is noteworthy that a mutant of T7 RNA polymerase able to transcribe three times more slowly than the wild-type enzyme has been shown to yield about four times more β -galactosidase from an appropriate expression vector (Makarova *et al.*, 1995). The C43(DE3) mutation may also be helping to avoid uncoupling of transcription and translation, but, in addition to delaying the onset of transcription, it also appears to affect the folding and insertion of subunit b into the bacterial membrane. In C41(DE3) the *E. coli* F-ATPase b subunit accumulates in a form that is difficult to solubilize in the detergent lauryldimethylamine oxide, and it may be misfolded, whereas in C43(DE3) it is inserted into the membrane and can be readily extracted with the detergent (I. Arechaga, B.M. & J.E.W., unpublished results).

The expression levels of a variety of proteins (seven membrane proteins, ten globular proteins; see Table 1) in BL21(DE3) were compared with the levels achieved in either C41(DE3) or C43(DE3) hosts. For all seven of the membrane proteins, and particularly for the alanine-H⁺ transporter and the *E. coli* F-ATPase subunits b and c, expression in the mutant hosts was improved over BL21(DE3). In all three of these latter examples, the induction of the expression both on plates and in liquid media was toxic to C41(DE3) but not to C43(DE3). The gene 10a-alanine-H⁺ carrier fusion was very well expressed in C43(DE3), and 79 mg of protein were obtained per litre of culture (Table 1). Significant improvements in expression level were also obtained with the ADP/ATP and phosphate carriers in C41(DE3).

A general improvement in the expression of the globular proteins (see Table 1) was also found in mutant host C41(DE3), including proteins that were well expressed as well as others that were poorly expressed in BL21(DE3). The GFP provides a typical example of the former category. Although it was expressed at 37 mg per litre of culture in BL21(DE3), a four times higher level of expression was obtained in C41(DE3) (see Table 1). The γ -subunit of bovine ATPase provides an example of the second category. Cells of BL21(DE3) containing pMW7(γ) stopped growing at low density, and the γ -subunit was undetectable by SDS-PAGE analysis of the cells, whereas in C41(DE3) the cells continued to divide, grew to high density and produced a large amount of the γ -subunit (see Table 1).

The RNA polymerase of phage T7 is inhibited by the phage's lysozyme (Moffatt & Studier, 1987), and therefore, co-transformation of a plasmid encoding the lysozyme (pLysS and pLysE) with the plasmid containing the target protein has been advocated as a means of suppressing toxic effects brought about by basal level expression of proteins (Studier *et al.*, 1990). This stratagem has been found to be helpful in some cases of relatively mild toxicity. However, co-transformation of pLysS with pMW7(γ) or with pMW7(GFP) did not suppress their toxicities on agar plates in the presence of IPTG. In liquid media, the level of expression of both proteins was somewhat higher in BL21(DE3) in the presence of pLysS than in its absence, but the level of expression of both proteins in C41(DE3) was at least twice the level obtained in co-transformed BL21(DE3) cells. Therefore, at least in these two examples, C41(DE3) is superior to BL21(DE3)-pLysS as a host for over-expression of proteins. The combination of C41(DE3) with pLysS may be advantageous, but this possibility has not been investigated systematically so far.

To date, the usage of strain C43(DE3) for expression of other toxic proteins has been explored in only a small number of examples (the F-ATPase b and c subunits, the alanine-H⁺ carrier). One example (an RNase) has been encountered where an expression vector that was toxic in C41(DE3) remained toxic in C43(DE3) also (unpublished results). A number of other examples show that, if the protein is already expressed without toxic effect in C41(DE3), then no additional benefit derives from over-expression of the same protein in C43(DE3). Therefore, it appears that C43(DE3) has a more restricted utility than C41(DE3).

Although the selection procedure presented here is empirical, it has the advantage that it encompasses the entire complexity of the biology of the expression system, and it has provided an efficient means of modifying it. The method takes advantage of a population of bacteria selected in the presence of both IPTG and ampicillin, which had been described incorrectly as only containing cells that have lost the ability to express the target DNA (Studier *et al.*, 1990). As we have shown, two

sub-populations giving rise to large and small colonies are present, and the latter contains cells that over-express the target protein better than the original host. Therefore, the procedure allows the expression system to be adapted and optimized for the expression of a particular protein, and it may be beneficial in other instances (including both globular and membrane proteins) to use the selection protocol to select a wider range of host strains derived, for example, from BL21(DE3), C41(DE3) and C43(DE3). In this way it may be possible to tailor the expression system by selection and thereby, for example, to prevent the formation of inclusion bodies, and to overcome toxic effects of various severities and origins.

Finally, it should be noted that removal of the toxic effects of an expression plasmid will not automatically guarantee that the protein is produced in large amounts, and to achieve this objective it may be necessary, for example, additionally to prevent mRNA degradation, to remove undesirable features in the coding sequence that impede translation (Kane, 1995), or to prevent proteolytic degradation.

Acknowledgements

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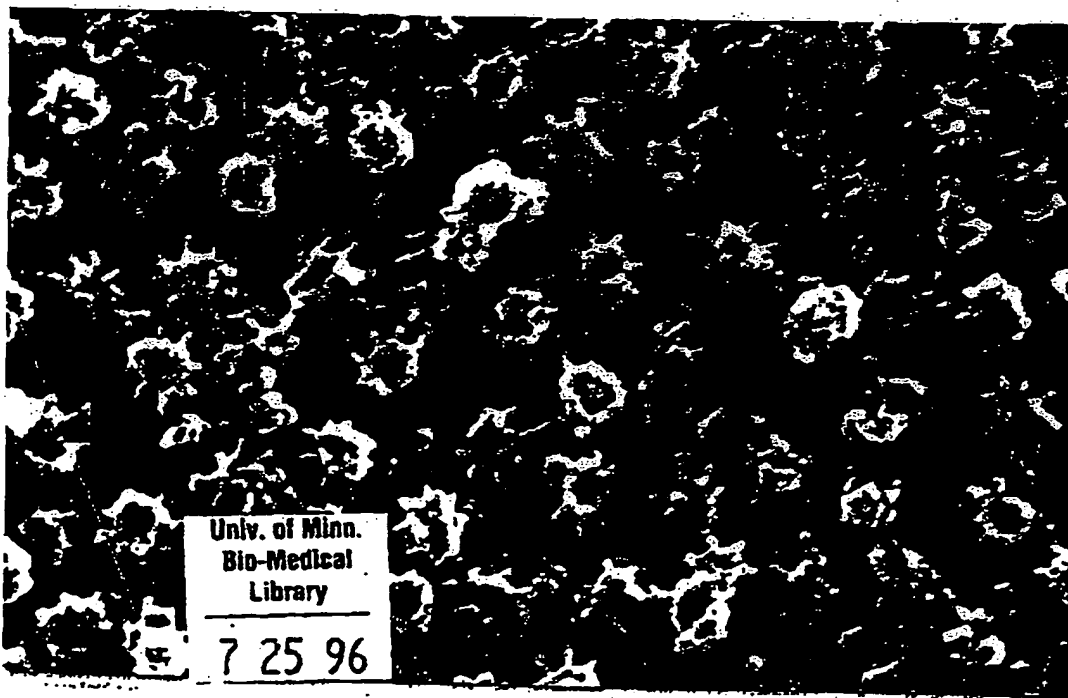
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